

Detection of Hydroxy-Nitro-PAHs
(-Polycyclic Aromatic Hydrocarbons) in an
Ambient Air Particulate Extract Using
Bioassay Directed Fractionation

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16. ABSTRACT Urban air particles contain extractable organic matter which has both mutagenic and carcinogenic activity. Detection of the specific compounds responsible for mutagenic activity has been limited by the complexity of these extracts and the presence of highly polar components which have been difficult to identify. To facilitate identifications, these extracts must be separated into substantially less complex fractions and analyzed with newer analytical techniques. Bioassay-directed fractionation has been proposed as the most cost and time effective approach to identifying mutagenic compounds in complex mixtures by directing the analytical and identification research effort toward only significantly mutagenic fractions. This approach should be especially useful for the detection of heretofore unknown constituents of the matrix as the methods are not directed to or developed for specific compounds or compound classes.		
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DETECTION OF HYDROXY-NITRO-PAHs AND NITRO-PAHs IN AN AMBIENT AIR PARTICULATE EXTRACT USING BIOASSAY DIRECTED FRACTIONATION

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INTRODUCTION

Urban air particles contain extractable organic matter which has both mutagenic (1) and carcinogenic (2,3) activity. Detection of the specific compounds responsible for the mutagenic activity has been limited by the complexity of these extracts and the presence of highly polar components which have been difficult to identify. To facilitate identifications, these extracts must be separated into substantially less complex fractions and analyzed with newer analytical techniques. Bioassay-directed fractionation has been proposed (4) as the most cost and time effective approach to identifying mutagenic compounds in complex mixtures by directing the analytical and identification research effort toward only significantly mutagenic fractions. This approach should be especially useful for the detection of heretofore unknown constituents of the matrix as the methods are not directed to or developed for specific compounds or compound classes.

This paper reports for the first time identification of mutagenic hydroxylated nitro-polycyclic aromatic hydrocarbons (OH-NO₂-PAHs) in a neutral polar fraction of an ambient air particulate extract using bioassay directed fractionation. Parent NO₂-PAHs were also identified and quantified using bioassay-directed fractionation. The relationship of these two classes of compounds, NO₂-PAHs and OH-NO₂-PAHs, will be discussed in regard to mammalian cell metabolism and atmospheric transformation.

MATERIALS AND METHODS

Air Particles

The air particulate sample used in this study was collected in an industrial/residential community of greater Philadelphia over two weeks using a Massive Air Volume

Sampler (MAVS). The operation of the MAVS has been described elsewhere in detail (5). Only particles <1.7 μ m, collected on Stage III of the MAVS, were used for this study.

A 49.1 g sample of the air particles was extracted (Soxhlet) under yellow fluorescent lights for 24 hours with one 300 mL volume of methylene chloride and then for 6 hours with a second 300 mL volume of methylene chloride (Burdick and Jackson, Distilled-in-Glass quality). These methylene chloride extracts were combined and concentrated by rotary film evaporation to a final volume of 20 mL.

First Level of Fractionation

The total fractionation scheme which resulted in analysis of one highly mutagenic subfraction is shown in Figure 1. Boldface type, therein, indicates the sequential path taken for this research effort. The first level of fractionation was applied to the methylene chloride particulate extract. This separation, involving acid-base partitioning and open bed silica gel column chromatography, has been described in detail elsewhere (6). The separation produces an organic acid, organic base and five neutral compound fractions. The silica gel elution solvents correspond to the designation of the fractions collected.

Second and Third Levels of Fractionation

The second and third levels of fractionation applied to the methylene chloride silica gel fraction (MeCl₂) utilized normal phase high performance liquid chromatography (HPLC) (Zorbax SiO₂, 4.6 mm x 25 cm column, Dupont Instrument Co). A flow rate of 1 mL/min was maintained and the eluate was monitored for UV absorbance at 254 nm. The second level of separation, applied to MeCl₂, used a linear gradient of 2 percent methanol/98 percent methylene chloride (v/v) in hexane, with the more polar solvent mix increasing from 8 to 35 percent in hexane over 30 minutes. The third level of separation, applied to the third subfraction of the methylene chloride fraction (MeCl₂-C), used a linear gradient of 80 percent methylene chloride in hexane increasing to 100 percent methylene chloride in 30 minutes.

The second and third levels of fractionation applied to the hexane/benzene silica gel fraction have been described in detail elsewhere (7).

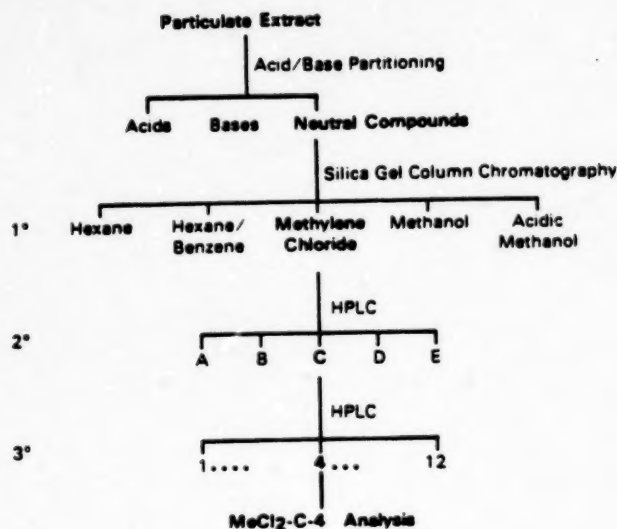


FIGURE 1. Overview of fractionation scheme.

Microbial Mutagenicity Method

The organic extracts and fractions were assayed for mutagenicity toward *Salmonella typhimurium* tester strain TA98 with and without exogenous metabolic activation (+S9,-S9) in the Ames plate incorporation assay (8). Minor modifications to this protocol, including the minimal histidine in the base agar and incubation of the plates for 72 hours is described in detail elsewhere (9). The studies reported here for TA98 included positive controls both with activation (2-anthramine) and without activation (2-nitrofluorene). Non-linear model slopes were determined from the dose response data (10).

Samples from the first level of fractionation were tested with and without S9 at seven doses with triplicate plates at each dose. Samples from the second level of fractionation were tested with S9 at 3-9 doses with single plates at each dose. Samples from the third level of fractionation were tested with S9 at a single dose in the range of 1-50 µg depending on available sample quantity.

EI and NCI HRGC/MS Method

Fractions were analyzed by combined gas chromatography/mass spectrometry (GC/MS) using a Finnigan Model 4500 quadrupole mass spectrometer equipped with a combined electron impact/chemical ionization (EI/CI) source. For EI analyses, a 70 eV electron beam was used. For NCI analyses, methane was used as the reagent gas at an uncorrected source pressure of 1.0 torr, with ionization at 150 eV. Data acquisition was controlled by an INCOS 2100 data system. For EI analyses, the mass range of 50-450 amu was scanned every 1 sec; for NCI analyses, the mass range of 100-450 amu was scanned every 1 sec.

High resolution gas chromatography (HRGC) for the EI and NCI mass spectral (MS) analyses was achieved using an Ultra #2 fused silica capillary column (5% phenylmethylsilicone bonded phase, 50M x 0.31 mm ID, 0.52 μ m film thickness; Hewlett Packard Co.) and methane carrier gas. The GC column was interfaced directly to the MS ion source. On-column injection at 380°C was used to prevent thermal degradation during sample introduction.

RESULTS AND DISCUSSION

Utility of Comparative EI and NCI HRGC/MS Analyses

Comparative EI and NCI HRGC/MS analyses were used in this study as a unique method to determine whether a complex fraction had been reduced in components to a relatively small number of potential mutagens. Comparative analysis was used first to determine whether additional fractionation was needed; second, to define the components of a subfraction; and third, to select compounds for mutagenicity testing.

The decision to continue fractionation or to begin chemical characterization of a subfraction was based on two criteria applied to the EI and NCI HRGC/MS data. When individual HRGC/MS chromatographic peaks contained a single component and the same set of compounds were detected in both analyses, further fractionation was not required. The criterion that peaks contain a single component assured unambiguous compound identification. The second criterion was chosen for the ability to detect different types of compounds by virtue of differing MS ionization mechanisms. While EI ionization provides approximately equal ionization

efficiency, and hence detection, for all compound classes, NCI greatly favors ionization, and hence detection, of highly electronegative compounds. Polar ambient air mutagens may be like nitro-PAHs, electronegative and present at low concentrations levels. Therefore, detection by the relatively non-selective MS technique (EI) of low level compounds which have been identified in the NCI analysis virtually precludes the possibility that other electronegative compounds have gone undetected. In this way the undetected components of an original fraction have become identifiable components of a subfraction and the comparative EI and NCI HRGC/MS analyses can be used to provide the definitive list of the components of that subfraction. In addition, the two mass spectral techniques provide complementary structural information; the NCI mass spectrum of a compound is dominated by a molecular ion and the EI mass spectrum contains more extensive fragment ion data.

As described above, the NCI conditions greatly favor detection of electronegative compounds. Since many such compounds are both chemically reactive and mutagenic (e.g., cyclopenta(c,d)pyrene, benzo(a)pyrene, 1-nitropyrene) we propose that NCI HRGC/MS analyses in detecting highly electronegative compounds may be, in addition to an identification technique, a selective method for identifying those compounds to be tested as potential mutagens.

Distribution of Mutagenicity Through Three Levels of Fractionation

The mass distribution and mutagenic activity for the seven fractions generated by the first level of fractionation of the air particulate extract have been detailed elsewhere (6). Mass recovery of 94 percent and mutagenicity recovery of 84 percent (-S9) and 88 percent (+S9) were achieved for this separation. The distribution of direct (-S9) and indirect (+S9) mutagenic activity among the compound class fractions of this separation are given in Table 1.

The organic acid and methylene chloride fractions contained the greatest proportion of the direct and indirect-acting mutagenicity, 38 and 45 percent, respectively. Isolation of almost 50 percent of the indirect-acting activity in only 9 percent of the extract mass indicated that the methylene chloride fraction (MeCl₂) should be given highest priority for detection of mutagenic compounds. Analysis of MeCl₂ by EI HRGC/MS showed that the most abundant

TABLE 1

DISTRIBUTION OF MASS AND MUTAGENIC ACTIVITY
FOR FIRST LEVEL OF FRACTIONATION

Fraction	Mass, %	Mutagenic Activity, % in TA98	
		-S9	+S9
Acids	7	38	21
Bases	1	1	1
Hexane	21	0	0
Hexane/Benzene	14	8	12
Methylene Chloride	9	23	45
Methanol	34	29	21
Acidic Methanol	14	1	<1

compounds belonged to compound classes which are not mutagenic toward TA98 (e.g., phthalate esters, aliphatic aldehydes). Due to the high concentration of these compounds little if any other material could be detected.

The optimized normal phase HPLC conditions for separation of MeCl₂ produced 86 percent mass recovery and the UV chromatogram seen in Figure 2. MeCl₂ was separated into the five subfractions (MeCl₂-A, MeCl₂-B, etc.) indicated on that chromatogram. For each subfraction, the percentage of recovered mass and of recovered mutagenicity (TA98, +S9 assay) are also indicated in Figure 2. On the basis of these data, fractions MeCl₂-C and MeCl₂-D were given highest priority for continued characterization. MeCl₂-C was considered most important as nearly 50 percent of the recovered activity was present in 25 percent of the mass.

Analysis of MeCl₂-C by both EI and NCI HRGC/MS techniques indicated that the HPLC separation had not reduced the complexity of the sample sufficiently. Individual peaks in the GC/MS total ion current (TIC) chromatograms were relatively broad and contained at least 4-6 distinct molecular ions with their overlapping fragment ion spectra. In addition, major components in the NCI analyses were not

DETECTION OF OH-NO₂-PAHS AND NO₂-PAHS

found in the EI analyses, suggesting the presence of additional compounds at lower levels. Further fractionation of MeCl₂-C was required.

Though considered a powerful tool for further separation, reverse phase HPLC of MeCl₂-C had to be rejected because highly colored, mutagenic material of the fraction was not soluble in the methanol/water solvent system used. However, normal phase HPLC conditions provided 85 percent mass recovery and sufficiently good peak shape and resolution to allow separation of MeCl₂-C into 12 fractions (MeCl₂-C-1, etc.) at reliable valleys in the chromatogram through multiple analyses. The UV chromatogram for this separation is shown in Figure 3; the mutagenic activity (TA98 +S9) of each fraction is included in this figure.

Those subfractions whose mutagenic activity was greater than the activity of the unfractionated material, MeCl₂-C, were given highest priority for further study. Subfractions fitting that criterion included MeCl₂-C-4 and MeCl₂-C-11, with activities of 29 and 37 rev/μg, respectively.

Detection of OH-NO₂-PAHS in Highly Mutagenic Subfraction MeCl₂-C-4

Mass spectral analysis of the MeCl₂-C-4 subfraction demonstrated that the goals of sequential fractionation had been achieved. The complexity of the original MeCl₂ fraction had been reduced to the point where most chromatographic peaks contained a single component. In addition, major components in the NCI HRGC/MS analysis were also detected in the EI HRGC/MS analysis. For comparative purposes, a selected portion of each analysis is displayed in Figure 4. The positions of two phthalate esters, butylbenzylphthalate (BBP) and di-2-ethylhexylphthalate (DEHP), were used to align these chromatograms for comparative purposes.

Hydroxy-nitro-substituted PAHS (OH-NO₂-PAHS), including a OH-NO₂-fluoranthene isomer, a OH-NO₂-fluorenone isomer, and two OH-NO₂-methylfluorenone isomers, were detected in these mass spectral analyses. The EI and NCI mass spectra for the detected OH-NO₂-fluorenone isomer are shown in Figure 5. The spectra of a reference OH-NO₂-PAH, 6-OH-1-NO₂-pyrene, acquired under identical conditions are shown in Figure 6. Both EI spectra show a strong molecular ion (base peak) and major fragment ions corresponding to the loss of O, NO, CNO₂

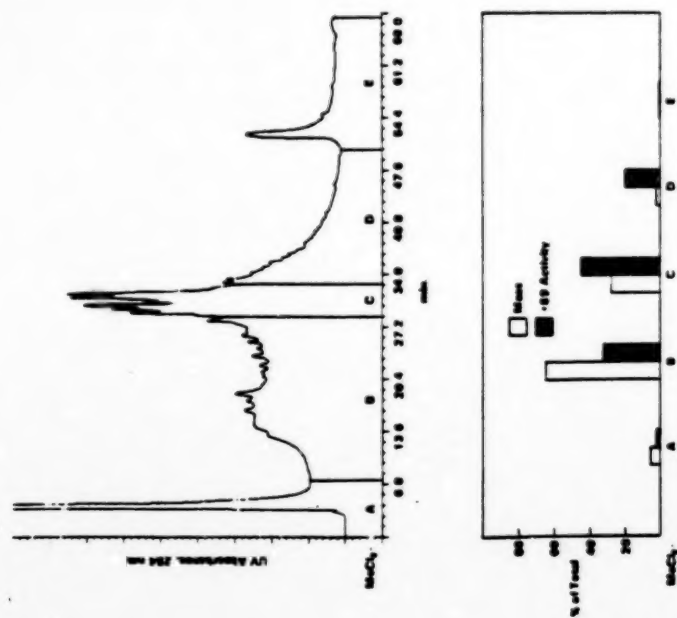


Figure 2. UV chromatogram for separation of MeCl₂ fraction. Percentage distribution of mass and mutagenic activity (TA 98, +S9) for 5 subfractions included.

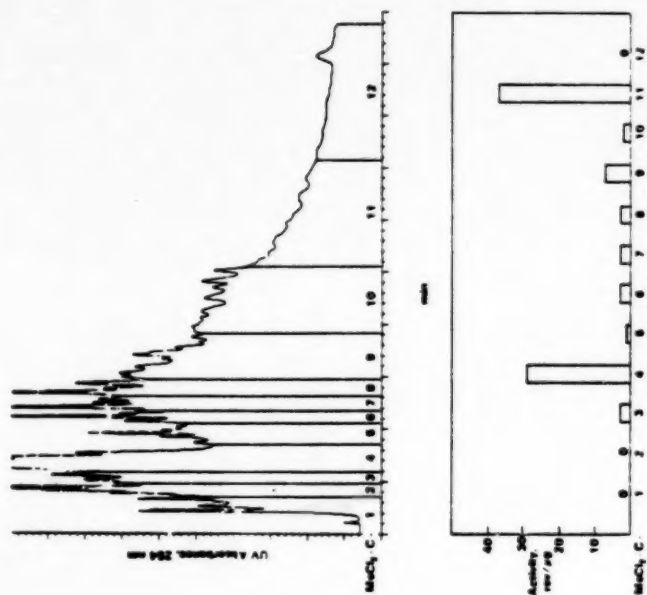


Figure 3. UV chromatogram for HPLC separation of MeCl₂-C fraction. Mutagenic activity (TA 98, +S9) for 12 subfractions included.

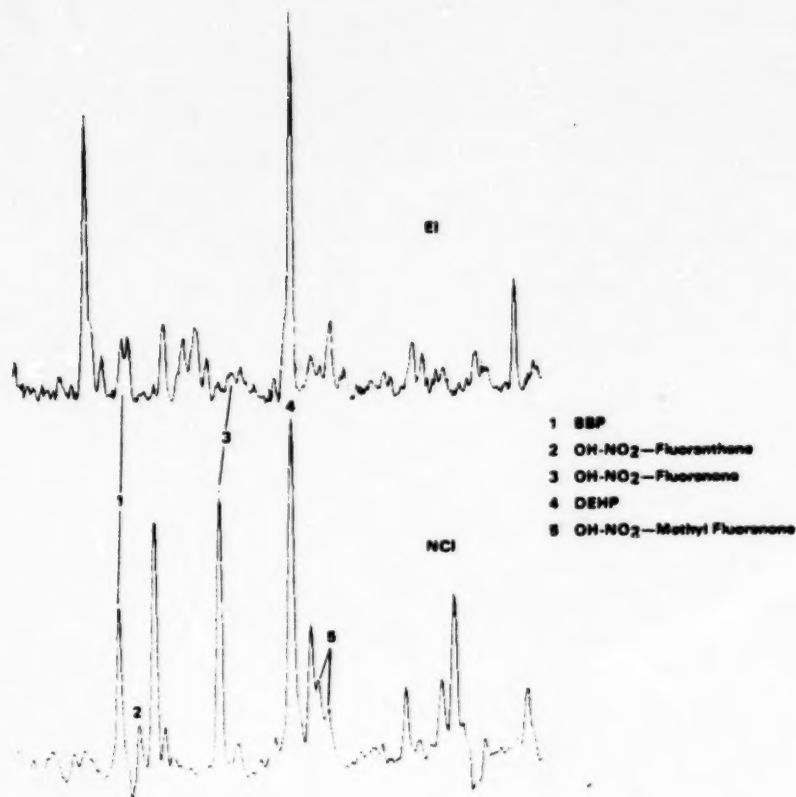


FIGURE 4. Comparative EI and NCI HRGC/MS analyses of MeCl₂-C-4.

major fragment ions corresponding to the loss of O, NO, CNO₂ and CONO₂ (M-16, M-30, M-58, and M-74 amu, respectively). Although the chromatographic peak for OH-NO₂-fluorenone in the EI analysis was quite small, the spectrum is unequivocal and contains no interferences other than the ion indicative of column bleed at m/z 281. The NCI spectra of the OH-NO₂-fluorenone isomer and reference 6-OH-1-NO₂-pyrene show strong molecular ions and fragmentation ions as indicated (M-16, M-30, M-32 and M-46), which are consistent with the structure. The NCI spectra of the detected OH-NO₂-fluoranthene and OH-NO₂-methylfluorenone isomers showed the same fragmentation pattern. Although these compounds were present at concentration levels too low for detection in the

EI analysis, their NCI spectra were sufficient for unequivocal identification.

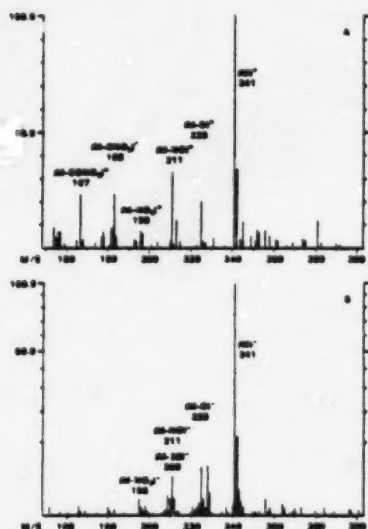


FIGURE 5. Mass spectra of OH-NO₂-fluorenone detected in MeCl₂-C-4. A-EI spectrum, B-NCI spectrum.

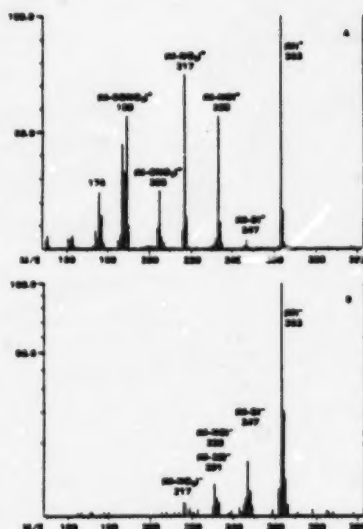


FIGURE 6. Mass spectrum of reference 6-OH-1-NO₂-pyrene. A-EI spectrum, B-NCI spectrum.

The MW 263 OH-NO₂-PAH detected in MeCl₂-C-4 has been identified as a OH-NO₂-fluoranthene isomer on the basis of chromatographic data. Available OH-NO₂-pyrene standards (1-NO₂-3-,4-,5-,6-,8-,9- and 10-OH) were analyzed by NCI HRGC/MS under chromatographic and mass spectral conditions identical to those used for the analysis of MeCl₂-C-4. Excellent chromatography was achieved for these compounds without derivatization of the OH substituent group. DEHP was selected as the retention marker for these analyses because, in addition to its presence in the MeCl₂-C-4 fraction, its close elution to the OH-NO₂-PAHs assured a more accurate isomer identification (based on RRT) than could be achieved with a structurally similar retention marker which eluted chromatographically far from the compounds of interest. The relative retention values for the detected OH-NO₂-

fluoranthene isomer and the standard OH-NO₂-pyrene isomers are listed in Table 2. The isomer detected in MeCl₂-C-4 eluted well ahead of DEHP; all OH-NO₂-pyrene isomers analyzed eluted later than DEHP. Although the 2 and 7-OH-1-NO₂-pyrene isomers were not available for analysis, neither one would be expected to elute earlier than 10-OH-1-NO₂-pyrene. Due to intramolecular hydrogen bonding, the 10-OH-1-NO₂ isomer is the least polar of the OH-NO₂-pyrene isomers. Therefore, on the basis of these relative retention values, and the fact that on this GC column phase fluoranthene elutes earlier than pyrene and 3-nitrofluoranthene elutes earlier than the nitropyrene isomers, we conclude that the OH-NO₂-fluoranthene/pyrene isomer detected in the MeCl₂-C-4 subfraction is a OH-NO₂-fluoranthene isomer.

TABLE 2.

RELATIVE RETENTION VALUES FOR REFERENCE OH-NO₂-PYRENE ISOMERS AND ISOMERIC OH-NO₂-FLUORANTHENE DETECTED IN FRACTION MeCl₂-C-4.

Compound	RRT(a)
OH-NO ₂ -fluoranthene in MeCl ₂ -C-4	0.89
10-OH-1-NO ₂ -pyrene	1.16
9-OH-1-NO ₂ -pyrene	1.25
3-OH-1-NO ₂ -pyrene	1.26
6-OH-1-NO ₂ -pyrene	1.27
8-OH-1-NO ₂ -pyrene	1.27
4-OH-1-NO ₂ -pyrene	1.27
5-OH-1-NO ₂ -pyrene	1.27

(a) Relative to DEHP.

Detection of Nitro-PAHs

Pitts has postulated that both NO₂-PAHs and OH-NO₂-PAHs in ambient air result from the same initial atmospheric reaction, hydroxylation of the parent PAH (11). For this reason the detection of a OH-NO₂-fluoranthene isomer, rather than a OH-NO₂-pyrene isomer, might not be unexpected as

3-NO₂-fluoranthene was present at higher concentration levels than 1-NO₂-pyrene (6.4 and 2.6 ug/g extract mass) in this extract. The detection of these NO₂-PAHs is detailed below. Subfractionation of the hexane/benzene silica gel fraction as detailed elsewhere (7), resulted in one highly purified PAH fraction (Pure PAH), a less polar aliphatic fraction and two fractions of greater polarity (Polar I, Polar II). Direct-acting activity in the Pure PAH fraction, suggestive of NO₂-PAHs, led to analysis for these compounds in the above subfractions. Nitro-PAHs were detected in the Pure PAH and Polar I subfractions. The concentrations of two of them, 3-NO₂-fluoranthene (3-NF) and 1-NO₂-pyrene (1-NP), are given in Table 3 as ug/g fraction mass. The concentration of benzo[a]pyrene (BaP) is included for comparison.

TABLE 3

CONTRIBUTION OF IDENTIFIED MUTAGENS TO ACTIVITY OF
HEXANE/BENZENE SUBFRACTIONS

Fraction	Activity, rev/ug -59					Activity, rev/ug +59		
		3-NF		1-NP			BaP	
		conc. ug/g	% MA	conc. ug/g	% MA		Conc. ug/g	% MA
Pure PAH	1.5	84	100	8	<1	4.1	7700	30
Polar 1	0.9	13	30	440	50	2.4	Not Analyzed	

The presence of 84 ug/g of the highly mutagenic (22,000 rev/ug) 3-NF could account for the direct-acting activity found in the Pure PAH fraction; approximately 80 percent of the direct-acting mutagenicity in Polar I could be accounted for by the presence of 1-NP (1000) rev/ug and 3-NF. These analyses indicated that the elution profiles of isomeric 3-NF and 1-NP were significantly different in the HPLC separation procedure used. While 1-NP was essentially isolated from the PAH by this technique, isomeric 3-NF remained with the PAH fraction. The significance of this separation difference is

seen when comparing the concentrations of BaP and 3-NF in the Pure PAH subfraction, i.e., 7700 µg/g vs. 84 µg/g, respectively. In spite of a concentration difference of approximately 100, the contribution of 3-NF to the direct-acting activity, 100 percent, was greater than the calculated contribution of BaP to the indirect-acting activity (~30 percent based on the mutagenicity of BaP at 168 rev/µg) of the Pure PAH fraction. Thus, even highly purified PAH fractions of environmental samples may contain mutagenic mono-nitro PAH (e.g., 3-NF) at concentrations which may account for the direct-acting mutagenicity often seen in those fractions.

Significance of OH-NO₂-PAHs and NO₂-PAHs

The importance of OH-NO₂-PAHs became apparent to us when OH-NO₂-pyrenes were identified as mutagenic mammalian metabolites of 1-NO₂-pyrene (1-NP) (12). The mutagenicity of those OH-NO₂-pyrene isomers tested (1-NO₂-3,4,5,6,8,9 and 10-OH) ranged from less than 1/10 (.06x) the activity of the parent 1-NP to 8 times the activity of 1-NP (13). Separation and isomer confirmation of these seven OH-NO₂-pyrene isomers (14,15) facilitated identification of the OH-NO₂-fluoranthene in this ambient air particulate extract.

The identification of 3-NF and now a OH-NO₂-fluoranthene isomer in ambient air particulate extracts suggests the importance of elucidating the potential health effects of these compounds. The higher mutagenic activity of 3-NF (22,000 rev/µg) versus 1-NP (1000 rev/µg) suggests that the hydroxylated derivatives of 3-NF may be more mutagenic than the OH-NO₂-pyrenes.

Disclaimer

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